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Evaluation of Tat-Encoding Bicistronic Human Immunodeficiency Virus Type 1 Gene Transfer Vectors in Primary Canine Bone Marrow Mononuclear Cells

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Tat-encoding human immunodeficiency virus type 1 (HIV-1) gene transfer vectors were evaluated in primary canine bone marrow mononuclear cells. Tat vectors provided higher levels of gene expression than vectors with internal promoters. The HIV-1 vector was also more efficient than Moloney murine leukemia virus (MoMLV) vectors for transduction of canine bone marrow mononuclear cells in vitro. Transplantation experiments in dogs with transduced autologous marrow cells confirmed the superiority of HIV-1 vectors over MoMLV vectors for gene transfer into canine bone marrow cells. Tat vectors may be useful not only for providing high levels of therapeutic gene expression in hematopoietic cells but also for study of the biological effects of Tat in those tissues in the canine model.

The human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) in the presence of its transcriptional activator, Tat, is one of the strongest promoters known. The Tat protein is encoded in two exons. The size of the Tat protein varies between different HIV-1 isolates and ranges from 86 to 101 amino acids (11). The first 72 amino acids of the Tat protein (Tat72) are encoded by the first coding exon. Tat72 is sufficient for transcriptional activation and for gene transfer (8, 19, 25). Tat72 can also recruit histone acetyltransferases (HATs) to the viral promoter (4, 14, 33). Recent work has revealed that this activity is important to ensure transcription from integrated vectors but not for expression with HIV-1 LTR promoter-reporter constructs in transient transfection assays (4). The ability of Tat to recruit HATs to viral promoters may prevent the diminution of gene expression due to chromatin remodeling that may occur with cellular differentiation or during prolonged in vitro culture of transduced cells. Other studies have indicated that Tat expression in cells can interfere with antigen presentation (5, 9, 10, 12, 31). Although these attributes make Tat-encoding vectors attractive for gene transfer, some biological effects of Tat, such as induction of apoptosis and its possible involvement in the genesis of Kaposi's sarcoma, render such vectors less suitable for gene therapy. Studies have revealed that while the first coding exon is sufficient for transcriptional activation, HAT recruitment, and gene transfer (8, 25), induction of Kaposi's sarcoma and apoptosis requires the second coding exon of Tat (3, 18, 32). Thus, Tat72-encoding vectors may have some of the necessary functionality, without the untoward effects of the 2-exon or 86-amino-acid Tat (Tat86), for gene therapy.

We have described Tat72- and Tat86-encoding HIV-1 vectors previously (26) (Fig. 1). pN-GIT72 is a bicistronic HIV-1 vector that encodes the enhanced green fluorescent protein (EGFP) gene and Tat72. Tat72 is translated by using the internal ribosome entry site (IRES) derived from encephalomyocarditis virus. pN-GIT86 is similar to pN-GIT72 but contains a 2-exon Tat (Tat86) instead of Tat72. These vectors have been shown to produce higher levels of transgene expression in a wide variety of cell lines, including cultured canine cell lines, than vectors that express the transgene under the control of the simian cytomegalovirus (sCMV) immediate-early promoter or the simian virus 40 (SV40) early promoter. In the present study, we have compared the different HIV-1 vectors with Moloney murine leukemia virus (MoMLV) vectors for transduction of primary canine bone marrow mononuclear cells (MNC) in order to evaluate these vectors for possible application in gene therapy experiments using the canine model.

To directly compare the HIV-1 vectors to MoMLV vectors, we created MoMLV vectors that expressed EGFP under the control of the MoMLV LTR or the SV40 early or sCMV immediate-early promoter-enhancer elements. The MoMLV vectors are based on the LgGC vector (kindly provided by Dusty Miller, University of Washington, Seattle). The LgGC vector contains the glutamine tRNA primer binding site instead of the proline tRNA site of the MoMLV-based LXSN vector (GenBank accession no. M28246) but is otherwise similar to LXSN (D. Miller, personal communication). The glucocerebrosidase (GC) gene in LgGC was replaced with the sCMV-EGFP or SV40-EGFP transgene expression cassette to create pLg-sCMV-GFP and pLg-SV-GFP, respectively. To express transgenes under the control of the MoMLV LTR, an EGFP-IRES-Neo cassette was introduced in place of the GC gene in LgGC to create pLg-eEIN. Vesicular stomatitis virus G (VSV-G) glycoprotein-pseudotyped HIV-1 and MoMLV vector stocks for each of the vectors were prepared by multiplasmid transfection of 293T (human embryonic kidney) cells as described previously (25, 26). Briefly, HIV-1 vector stocks were prepared by using the packaging plasmid pCMVΔR9 (17), the VSV-G-expressing plasmid pMD.G (17), and one of the gene transfer vectors shown in Fig. 1. MoMLV vectors were pre-

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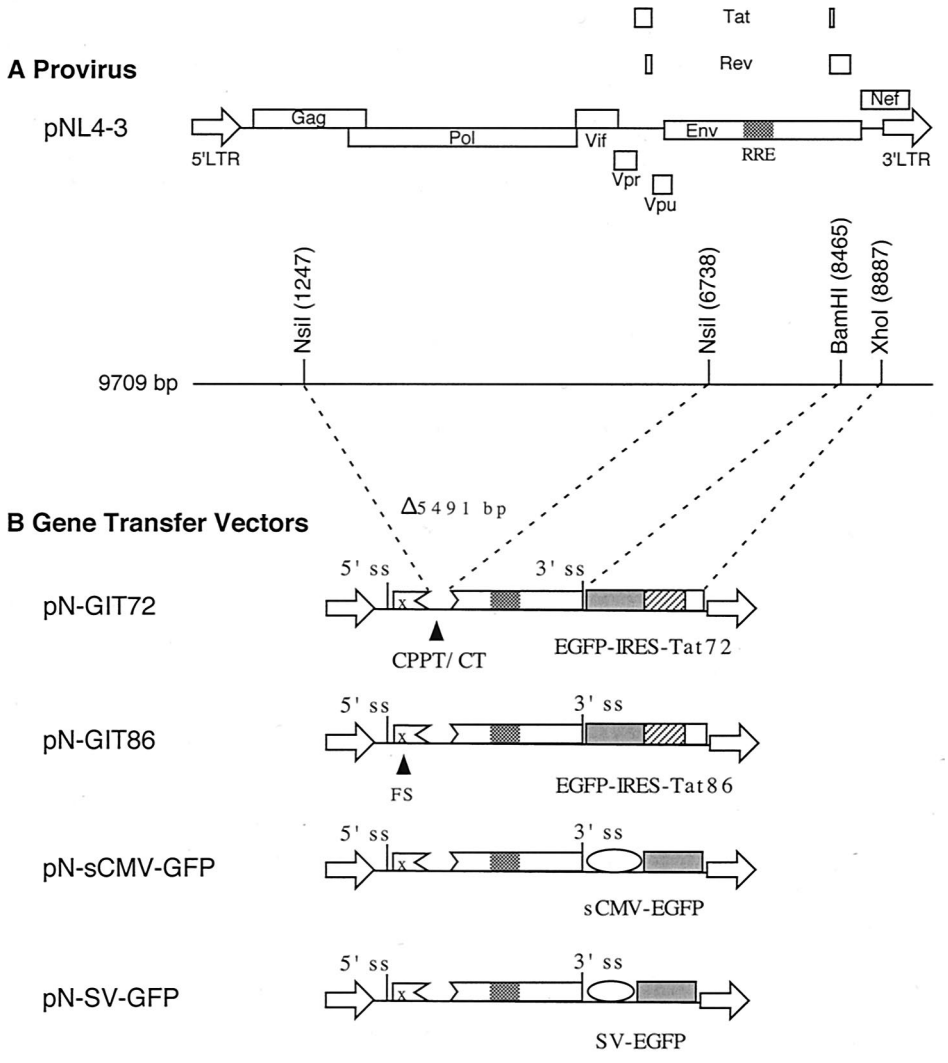

FIG. 1. Schematic representation of HIV-1 provirus (A) and gene transfer vectors (B). The HIV-1 vectors are based on the molecular clone pNL4-3 (GenBank accession no. M19921). The gene transfer vectors express the EGFP gene under the control of the HIV-1 LTR, the sCMV immediate-early promoter (bp 681 to 1349 of the IE94 gene [GenBank accession no. M16019]), or the SV40 early promoter (bp 5175 to 5243 and 1 to 272 of SV40 [GenBank accession no. J02400]). The restriction enzyme sites in the provirus that are used for creation of the gene transfer vectors are shown. The vectors contain a frameshift (FS) mutation in the *gag* open reading frame between codons 9 and 10 by insertion of an A residue (indicated by a lowercase "x"). All transgene expression cassettes were positioned downstream of the 3′ splice acceptor site of Tat and Rev between the *Bam*HI site in the second coding exon of *rev* and the *Xho*I site in *nef*. The EGFP gene was introduced upstream of the IRES-Tat cassette between unique *Bam*HI and *Not*I restriction enzyme sites or downstream of the sCMV or SV40 promoter between unique *Bam*HI and *Xho*I sites. Tat72 and Tat86 were translated via an encephalomyocarditis virus IRES (bp 361 to 860 of encephalomyocarditis virus [GenBank accession no. X74312]). The vectors have been described elsewhere [26]. RRE, Rev response element; 5′ ss and 3′ ss, 5′ and 3′ splice donor and acceptor sites, respectively.

pared by cotransfection of pSV-A-MLV-GagPol (13), pMD.G, and one of the Moloney gene transfer vectors described above. The vector stocks were concentrated 100- to 200-fold by ultracentrifugation and titered on D17 (canine osteosarcoma) cells or 293 cells. Flow cytometry of indicator cells infected with vector stocks (26) showed that they had comparable titers of about 10^8 infectious units (IU)/ml.

Bone marrow was aspirated from the humeri of healthy beagles and hounds, and MNC were obtained by centrifugation on Histopaque (Sigma, St. Louis, Mo.) gradients. The interface containing the MNC was harvested, and the cells were washed and resuspended in phosphate-buffered saline (PBS) prior to use as targets for infection or for further enrichment for granulocyte-macrophage CFU (CFU-GM) progenitors by centrifugation on discontinuous albumin density gradients (ADG) (22). For cytokine prestimulation, cells were incubated for 48 h at 37°C under 5% CO_2 in RPMI 1640 containing 20% horse serum, 10^{-6} M hydrocortisone-hemisuccinate, and the following combination of cytokines: human interleukin 1 (IL-1) (50 ng/ml), IL-3 (10 ng/ml), and IL-6 (50 ng/ml) and canine stem cell factor (cSCF) (50 ng/ml).

Freshly isolated MNC or MNC, stimulated with cytokines for 48 h, were placed in 6- or 24-well tissue culture plates (200,000 cells per well) and infected overnight with HIV-1 or

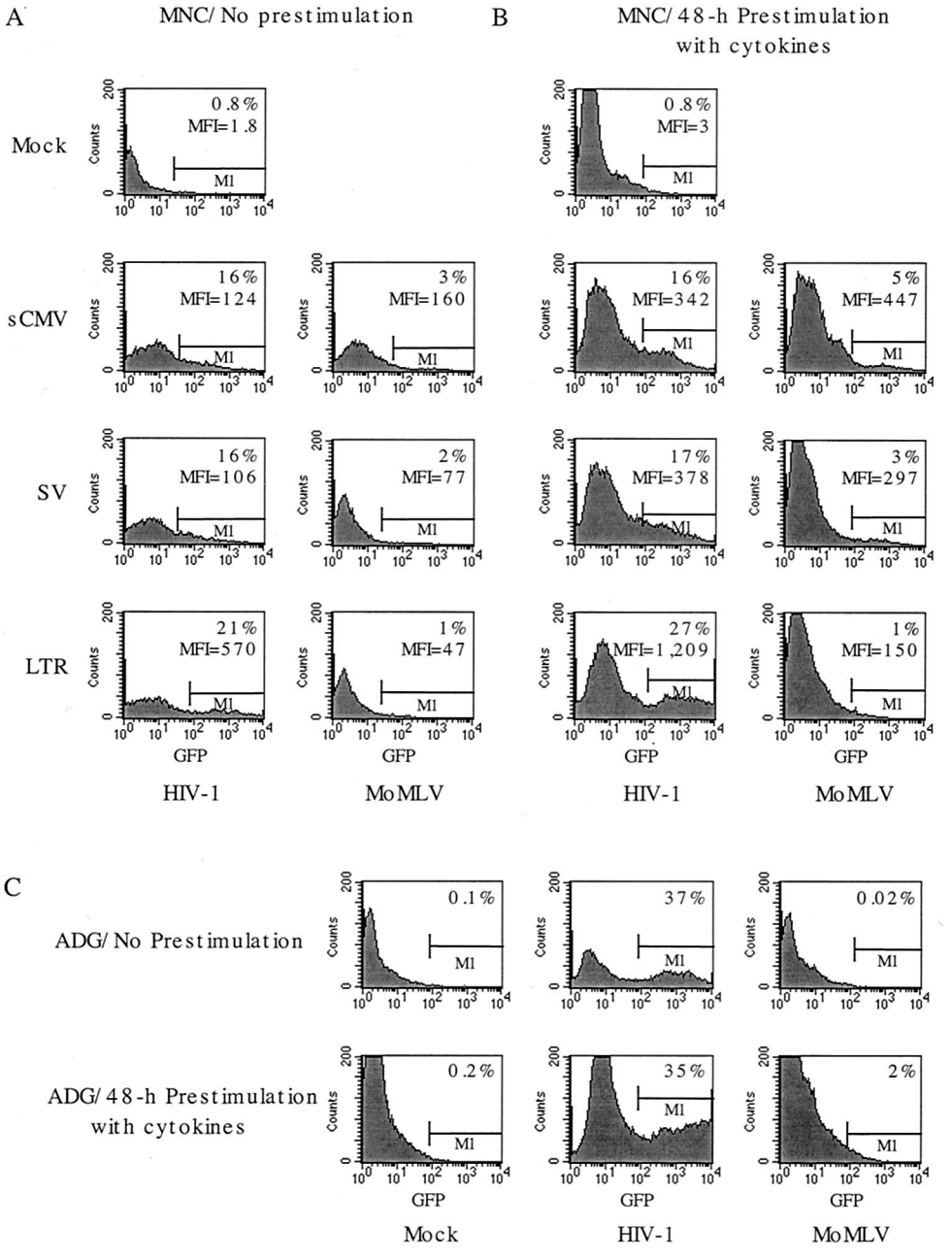

FIG. 2. Flow cytometry of canine bone marrow MNC transduced with HIV-1 or MoMLV vectors encoding EGFP under the control of the sCMV immediate-early, SV40 early, or viral LTR promoter-enhancer elements. Canine bone marrow MNC were either used fresh (A) or after prestimulation for 48 h in a medium containing a cytokine cocktail of human IL-1, IL-3, and IL-6 and cSCF (B), or MNC isolated on ADG (see the text), either fresh or after 48 h of cytokine prestimulation (C), were used as targets for transduction with the vectors. The cells were harvested 5 days postinfection and analyzed by flow cytometry. The percentage of cells expressing EGFP and the geometric MFI for the population (M1) are shown. In panel C, the vectors encoded EGFP under the control of the respective LTR promoters.

MLV vectors in 0.5 to 1 ml of medium containing 8 μg of Polybrene/ml. Infection was carried out at a multiplicity of infection (MOI) of 33 or 3. All wells received twice the initial volume of medium containing growth factors (human IL-1, IL-3, and IL-6 and cSCF) the following day. The cells were harvested 3 to 5 days after infection, fixed with 5% paraformaldehyde, and analyzed by flow cytometry. The results of infection at the higher MOI are shown in Fig. 2. The HIV-1 vector was found to transduce both unstimulated and cytokine-prestimulated canine bone marrow cells more efficiently than the MoMLV vector. Thus, the HIV-1 vector, at an MOI of 33, transduced 16 to 21% of unstimulated bone marrow MNC and 16 to 27% of bone marrow MNC prestimulated with a cytokine mixture for 48 h. In contrast, the MoMLV vectors transduced 1 to 3% of unstimulated targets and 1 to 5% of prestimulated targets. At a 10-fold-lower MOI of 3, transduction of cells was

TABLE 1. Transduction of CFU-GM progenitors by HIV-1 and MoMLV vectors

Vector	% EGFP$^+$ CFU-GM progenitors[a] with:	
	No prestimulation	48 h of prestimulation with cytokines
Expt 1		
Mock	0	0
pN-GIT72	7.5 ± 4.9	31.5 ± 10.4
pN-sCMVGFP	1.4 ± 1.4	15 ± 4.7
pN-SVGFP	0	13.3 ± 4.3
Lg-eEIN	0	0
Lg-sCMVGFP	2.7 ± 0.3	8.8 ± 3.2
Lg-SVGFP	0	12.9 ± 3.1
Expt 2		
Mock	0	0
pN-GIT72	4 ± 8	21 ± 7
pN-GIT86	5 ± 8	26 ± 12

[a] Means ± standard deviations from triplicate experiments.

reduced by about 2-fold. This is consistent with other recent reports that increasing the MOI results in a less-than-proportionate increase in transduction efficiency (16, 30). Consistent with our previous experience with cultured cell lines, the HIV-1 LTR provided the highest levels of gene expression, as measured by the geometric mean fluorescence intensity (MFI) of EGFP expression, in transduced cells. This probably contributed to the apparently higher efficiency of gene transfer by the Tat-encoding vector compared to those of vectors with internal promoters. We also tested the vectors on MNC that had been fractionated on ADG in order to enrich them for CFU-GM progenitors (22). This method provides an alternative approach to enrichment of hematopoietic progenitor cells. Between 35 and 37% of these cells could be readily transduced with the HIV-1 vector by a single exposure to vector stock. In contrast to these results with the HIV-1 vector, the MoMLV vector was able to transduce only 0.02 to 2.0% of the ADG-fractionated MNC.

A portion of the transduced MNC was subjected to colony-forming assays for CFU-GM progenitors to determine the efficiency of transduction into these progenitor cells within the bone marrow MNC, as a predictor of transduction into hematopoietic stem cells. For CFU-GM assays, unfractionated bone marrow MNC (75×10^3) or ADG-fractionated bone marrow MNC (20×10^3 to 75×10^3) were plated in triplicate in 35-mm culture dishes (Costar, Cambridge, Mass.) in 2 ml of Dulbecco's modified Eagle medium (Gibco BRL) containing 0.3% agar (Difco Laboratories, Detroit, Mich.), 20% heat-inactivated human AB plasma, and growth factors (50 ng of IL-1 [Syntex Laboratories, Inc., Palo Alto, Calif.]/ml, 10 ng of IL-6/ml, 5 ng of IL-3 [Promega, Madison, Wis.]/ml, 50 ng of cSCF/ml, 50 ng of canine granulocyte-macrophage colony-stimulating factor/ml, and 50 ng of canine granulocyte–colony-stimulating factor [Immunex Corp., Seattle, Wash.]/ml). Cultures were maintained in a 100% humidified atmosphere at 37°C and 5% CO_2. Colonies were enumerated under a stereomicroscope after 14 days. The percentage of EGFP-positive colonies was determined.

The results of these studies are shown in Table 1. When bone marrow cells that were not prestimulated with cytokines were used as target cells, the HIV-1 vectors were able to transduce 0 to 7.5% of CFU-GM while the MoMLV vector could transduce only 0 to 2.7% of CFU-GM, depending on the type of promoter used. For both vectors, transduction of CFU-GM progenitors was enhanced by prestimulation of the bone marrow cells with cytokines for 48 h prior to infection. Thus, preactivation of MNC with cytokines resulted in 13 to 31% of CFU-GM colonies exhibiting EGFP expression when the HIV-1 vector was used. For the MoMLV vector, 0 to 13% of CFU-GM colonies were EGFP positive. Again, the differences between the Tat-encoding vector and vectors with internal promoters may be the result of higher levels of EGFP expression by the Tat vector, thus facilitating the enumeration of EGFP-expressing colonies. The results, nevertheless, indicate that HIV-1 and MoMLV vectors can transduce canine CFU-GM progenitors and that cytokine prestimulation enhances transduction into this population. The results also demonstrate that the HIV-1 Tat protein can function in primary canine hematopoietic progenitor cells.

Comparison of Tat72- versus Tat86-encoding HIV-1 vectors for gene transfer into canine bone marrow MNC. To determine if there were differences between Tat72 and Tat86 vectors for transduction and expression of marker genes in canine primary bone marrow MNC, separate virus stocks of Tat72 and Tat86 vectors, encoding the EGFP transgene, were used for infection of either freshly prepared or cytokine-prestimulated canine bone marrow MNC. Cells were harvested 5 days after infection, fixed with paraformaldehyde, and analyzed by flow cytometry.

The results of a representative experiment are shown in Table 2 (experiment 1). They indicate that Tat72 and Tat86 vectors provided similar levels of gene transfer into primary canine bone marrow MNC, with a slightly higher percentage of transduced cells for the Tat72 vector (35 to 48% for Tat72 versus 28 to 39% for Tat86 vectors). This was true both for freshly isolated cells and for 48-h cytokine-prestimulated cells. Cytokine-prestimulated cells showed slightly higher levels of transduction than unstimulated cells.

A portion of the transduced MNC were plated for CFU-GM, and the percentage of EGFP-positive colonies was determined after 14 days by counting under a fluorescence microscope. In parallel, we also tested the vectors on canine bone marrow MNC that were enriched for CFU-GM by centrifugation on ADG. These cells were transduced without cytokine prestimulation, and the following day, they were washed and plated for CFU-GM colony formation. The results of these experiments are shown in Table 1 (experiment 2). For cells that were not preactivated before transduction with Tat72 or Tat86 vectors, 4 to5% of CFU-GM colonies were EGFP positive. In contrast to these results, 21 to 26% of CFU-GM colonies were transduced when cytokine-prestimulated cells were used. Again, preactivation with cytokines resulted in increased transduction of CFU-GM progenitors by HIV-1 vectors.

The results described above indicate that Tat72 and Tat86 vectors provide similar levels of transduction of both CFU-GM progenitors as well as bone marrow MNC. Since CFU-GM colonies remained EGFP positive for approximately 2 weeks, this indicates that expression of Tat in hematopoietic progen-

TABLE 2. Comparison of transduction and expression of EGFP in canine MNC by Tat72 and Tat86 HIV-1 vectors and a vector with an internal PGK promoter

Vector	No prestimulation			48-h prestimulation		
	% GFP$^+$ cells	MFI	Fold increase in MFI	% GFP$^+$ cells	MFI	Fold increase in MFI
Expt 1						
Mock	0.1	4		0.1	4	
pN-GIT72	35.1	362	1	48.2	650	1
pN-GIT86	27.9	355	1	38.8	559	0.9
Expt 2[a]						
Mock	0.25	1.4		0.15	1.3	
pN-PGK-GFP-WPRE/CPPT	2.3	612	1	22.9	358	1
pN-GIT72/CPPT	7.7	1,434	2.3	30.5	1,440	4.0
pN-GIT86/CPPT	5.9	1,307	2.1	27.3	1,975	5.5

[a] Vector stocks used in experiment 2 were prepared by multiplasmid transfection of 293T cells using the packaging plasmid pCDNA-gp-RRE, pCMVTat, pCMVrev, pMD.G, and the indicated gene transfer vector. pCDNA-gp-RRE contains the HIV-1 Gag/Pol and RRE sequences under the control of the human CMV immediate-early promoter in plasmid vector pCDNA3 (Invitrogen Corp., Carlsbad, Calif.). The packaging plasmid lacks all accessory protein coding sequences (details about this construct will be provided on request). pCMVTat and pCMVRev express the Tat and Rev proteins of HIV-1 under the control of the sCMV immediate-early promoter (24a).

itors was not inimical to their differentiation. However, we cannot rule out the possibility that Tat may have subtly altered the functioning of the cells. This can be ascertained only by more extensive experimentation, including transplantation and follow-up of the transduced cells in vivo.

Effects of including CPPT and CT sequences on gene transfer. Recent reports indicate that the central polypurine tract (CPPT) and termination (CT) sequences are important for creation of a DNA flap during the reverse transcription process (6). These sequences have been shown to be critical for importation of preintegration complexes (PICs) into the nuclei of dividing and also nondividing cells (7, 23, 24, 34). Some of the vectors we described above lacked the CPPT and CT sequences. We wished to determine if addition of these sequences would enhance gene transfer into canine bone marrow MNC. To this end, we created HIV-1 gene transfer vectors that contained the CPPT and CT sequences in either a sense or an antisense orientation. Separate vector stocks were prepared for vectors with or without the CPPT and CT sequences, and equivalent amounts of vector stocks (based on p24) were used for infection of freshly isolated bone marrow cells or canine bone marrow MNC stimulated for 48 h with cytokines. The cells were harvested 5 days posttransduction and analyzed by flow cytometry as described for Fig. 2. The results of this experiment are shown in Fig. 3. In agreement with other reports (7, 23, 24, 34), inclusion of CPPT and CT sequences resulted in enhanced levels of gene transfer into canine bone marrow MNC. This was true both for freshly isolated cells and for cells that were prestimulated for 48 h with cytokines.

Comparison of Tat vectors with newer HIV-1 vectors. We compared the Tat vectors to a vector that expressed the EGFP gene under the control of a constitutively active cellular promoter—the mouse phosphoglycerate kinase (PGK) promoter. The mouse PGK promoter has previously been shown to function efficiently in human CD34$^+$ cells (21). The HIV-1 vector with the PGK promoter is similar to vectors with sCMV or SV40 promoters. This vector was further modified by including the woodchuck posttranscriptional regulatory element (WPRE), which has previously been shown to enhance transgene expression five- to eightfold (35). All the vectors, including the Tat vectors, were modified by inclusion of the CPPT and CT sequences (see below). Separate stocks of each of the different vectors were then used for infection of unstimulated or 48-h cytokine-prestimulated canine MNC. The results of this experiment are shown in Table 2 (experiment 2). The Tat vectors provided two- to sixfold-higher levels of EGFP expression (as measured by the MFI of GFP-positive cells) than the vector with the PGK promoter. Thus, the Tat vectors, in our hands, provided levels of transgene expression as high as or greater than the levels achieved with the current generation of vectors with internal promoters.

Autologous transplantation of ex vivo-transduced canine bone marrow cells. Encouraged by the in vitro transduction data, we carried out autologous transplantation of ex vivo-transduced canine bone marrow cells in three dogs. Animal experimentation complied with all relevant federal guidelines and institutional policies. CD34$^+$ bone marrow MNC were isolated (15) and exposed to vector-containing supernatants. Half of the bone marrow cells were transduced with an HIV-1 vector, while the other half were transduced with the corresponding MoMLV vector. No cytokines were used before or during the transduction procedure, in order to avoid possible irreversible differentiation into committed progenitor cells. The cells were harvested after 18 to 20 h of incubation with the vector supernatant, mixed, and injected via a peripheral vein into the bone marrow donor, who had been previously irradiated with a nonmyeloablative dose (200 cGy) of total-body irradiation (TBI). Dog 254 received CD34$^+$ cells transduced with HIV-1 and MoMLV vectors that encoded the EGFP transgene under the control of the respective LTR promoters. Dog 293 received cells transduced with vectors that encoded EGFP under the control of the SV40 early promoter, and dog 354 received CD34$^+$ cells transduced with vectors that contained the sCMV immediate-early promoter. The HIV-1 vector used for dog 254 also contained the CPPT and CT sequences, while those for dogs 293 and 354 did not contain these sequences. A portion of the cells used for transduction was analyzed by flow cytometry to determine transduction efficiency. The results of the flow cytometry analysis are shown in Table 3. Consistent with our previous in vitro data using bone

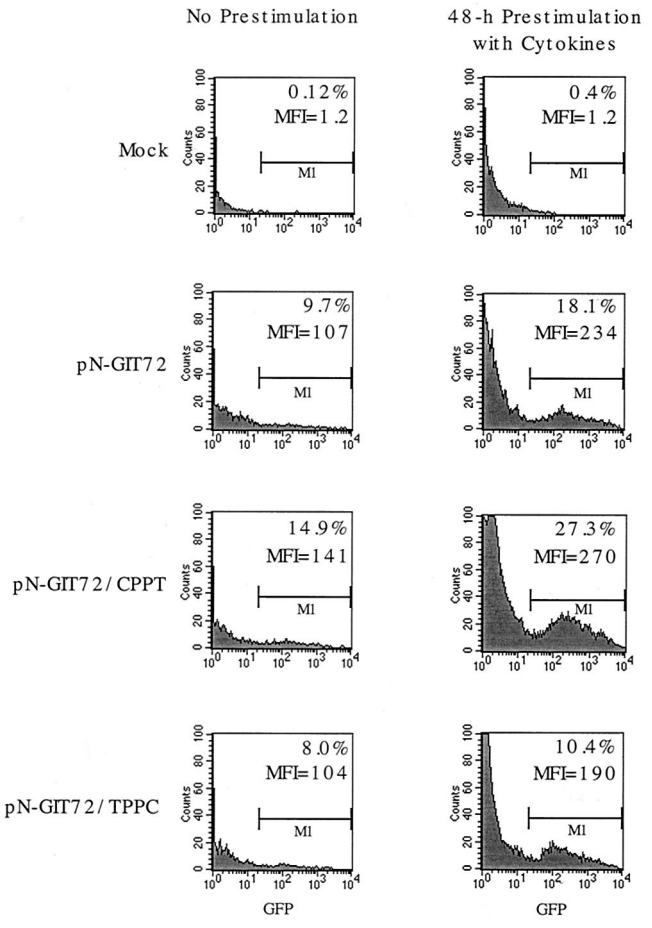

FIG. 3. Flow cytometry of canine bone marrow MNC transduced with Tat72 vectors containing the CPPT and CT sequences in the sense (pN-GIT72/CPPT) or antisense (pN-GIT72/TPPC) orientation or lacking the sequences (pN-GIT72). Cells with (right) or without (left) cytokine prestimulation were used for transduction. The percentage of cells expressing EGFP and the MFI for the population (M1) are shown.

marrow MNC, HIV-1 vectors proved to be more efficient than MoMLV vectors for transduction of primary canine bone marrow-derived $CD34^+$ cells.

Following transplantation, peripheral blood samples were obtained weekly for up to 24 weeks and bone marrow samples were obtained monthly. Genomic DNA was isolated from peripheral blood granulocytes, lymphocytes, and monocytes and from bone marrow MNC and was subjected to semiquantitative PCR to detect the presence of vector genomes. For HIV-1, the following primer pairs were used. For the first PCR, the sense primer SK47 (9076-TGG AAG GGC TAA TTC AC-9092) and the antisense primer SK48 (680-CGA GTC CTG CGT CGA GAG ATC-700) were used with commercial buffers and *Taq* polymerase (Promega). For the nested PCR, the same sense primer (SK47) was used with the antisense primer SK46 (652-TGG CTT TAC TTT CGC TTT CAA GT-674). The numbers correspond to nucleotide numbers in pNL4-3 (GenBank accession no. M19921). The sense primer corresponds to the sequence present in the U3 of the 3′ LTR and was designed in such a way that it would only prime off of the 3′ U3 sequence. The antisense primers correspond to the sequence in the 5′ untranslated region upstream of *gag*. Since the the U3 region of the 3′ LTR is copied and positioned in the 5′ LTR after reverse-transcription, this ensured that PCR amplified only vector sequences after the reverse transcription step and overcame false-positive PCR amplification as a result of contamination with plasmid DNA. Aliquots (200 ng) of genomic DNA derived from normal canine bone marrow MNC, spiked with different amounts of genomic DNA transduced with an HIV-1 vector and used for generation of the standard curve for quantitation of HIV-1 vector copy numbers, served as the positive controls. The 200-ng sample of genomic DNA derived from normal canine bone marrow MNC, without any added HIV-1 vector-transduced D17 genomic DNA, served as the negative control. For the first round of PCR, the following cycling conditions were used: an initial cycle of 95°C for 3 min, followed by 30 cycles of 47°C for 30 s, 72°C for 30 s, and 95°C for 30 s, and a final cycle of 47°C for 30 s and 72°C for 5 min. The conditions for the nested PCR were as follows: one step at 95°C for 3 min, followed by 25 cycles of 47°C for 30 s, 72°C for 30 s, and 95°C for 10 s, and a final cycle of 47°C for 30 s and 72°C for 5 min.

For amplification of the MoMLV vector, for the first PCR, the sense primer MLV_LTR (5′-GAA GTT CAG ATC AAG GTC AGG-3′) and the antisense primer A-MLV-psi3 (5′-TAG ACA ATC GGA CAG ACA CAG-3′) were used. For the nested PCR, the sense primer S-MLV_LTR2 (3021-5′-GGT CAG GAA CAG ATG GAA CAG C-3′-3042) and the antisense primer A-MLV_psi4 (5′-CAG GGG TCT CCA AAT CTC GG-3′) were used. The sense primers corresponded to sequences upstream of the unique *Xba*I site in the 3′ LTR, and the antisense primers corresponded to sequences present in the packaging-encapsidation sequence in the 5′ untranslated region. Positive PCR products were digested with *Xba*I to validate the authenticity of the product. Known amounts of control genomic DNA, derived from CTAC cells transduced with an MoMLV vector, were used as standards. For the first round of PCR, the following cycling conditions were used: an initial step at 95°C for 3 min, followed by 30 cycles of 57°C for 30 s, 72°C for 30 s, and 95°C for 30 s, and a final cycle of 47°C for 30 s and 72°C for 7 min. The conditions for the nested PCR were as follows: one cycle of 95°C for 3 min, followed by 25 cycles of 57°C for 30 s, 72°C for 30 s, and 95°C for 10 s, and a final cycle of 47°C for 30 s and 72°C for 7 min.

To amplify a control canine cellular gene, we used primers specific for canine IL-8. The sense primer S-cil8 (5′-CTC TCT TGG CAG CTT TTG TCC-3′) and the antisense primer A-cil8 (5′-GCA TCT CAC ACC TGA TTT AGG C-3′) were used for PCR amplification. The following conditions were

TABLE 3. Summary of results of transduction experiments using canine bone marrow $CD34^+$ cells with HIV-1 and MoMLV vectors

Dog no.	Promoter	MOI	% of cells transduced by:		No. of $CD34^+$ cells transplanted (10^6)
			HIV-1	MoMLV	
254	LTR	9.0	12	3	5
293	SV40	2.0	2.1	1.3	30
354	CMV	8.0	9.4	2.9	13

used for amplification of the IL-8 sequence: an initial cycle of 95°C for 5 min, followed by 35 cycles of 63°C for 30 s, 72°C for 1 min, and 96°C for 10 s, followed by incubation at 63°C for 30 s and then by incubation at 72°C for 7 min.

Although PCR analyses of both lymphocyte and granulocyte populations were carried out, only the results of PCR analyses with granulocytes are shown in Fig. 4, since there was no significant marking of lymphocytes. The data indicate the following. In dog 254, 0.003 to 3% of granulocytes were marked with the HIV-1 vector. Interestingly, the marked cells disappeared after 7 weeks. Marking by the HIV-1 vector was poor for dog 293. In dog 354, 0.01 to 0.1% of granulocytes were positive for the HIV-1 vector sequence. One lymphocyte sample at week 22 was also positive for the HIV-1 vector sequence. No marking by the MoMLV vectors was seen in any of the dogs. The highest levels of marking of peripheral granulocytes by the HIV-1 vector were seen in dog 254, which received cells transduced with a vector containing the CPPT and CT elements. We suspect that the lower levels of marking of cells by HIV-1 vectors in dogs 293 and 354 may be attributable to either the absence of CPPT and CT sequences in those vector constructs, the different MOIs used for transduction, the different numbers of cells used for transplantation, or a combination of these factors. In any case, this level of marking obtained with HIV-1 vectors was not seen in our previous experiments with MoMLV vectors after a single exposure to vector-containing supernatants in the absence of cytokine prestimulation. This is also, to the best of our knowledge, the first report for a large animal model where MoMLV and HIV-1 vectors were compared within the same animal.

The data also indicate that, under the conditions used, HIV-1 vectors seem to have transduced predominantly granulocyte precursors and that long-term repopulating cells may not have been transduced with the HIV-1 vector. Attempts at enumerating EGFP expression in various subpopulations in the peripheral blood were not successful, due to either low numbers of $EGFP^+$ cells in circulation (dogs 293 and 354), low levels of expression, or technical reasons (e.g., quenching of EGFP during fixation with paraformaldehyde).

An and coworkers reported on gene transfer into hematopoietic stem cells in rhesus macaques (1, 2). These investigators also found low levels of marking after transplantation of ex vivo-transduced $CD34^+$ cells derived from the bone marrow. Better marking was observed when the investigators used mobilized peripheral blood $CD34^+$ cells. With an EGFP-encoding vector, marking or EGFP expression was seen in 1 to 10% of different lineages of peripheral blood cells, including red blood cells and platelets. Using the common gamma-chain cytokine receptor transgene, the investigators found marking in 0.42 to 0.001% of peripheral blood cells. In that study, the authors also demonstrated that the choice of promoter was critical to obtaining high levels of EGFP expression in peripheral blood cells. A significant difference between our report and the previous study was that the monkeys received a myeloablative dose (10 Gy) of TBI while the dogs described in this study received only a nonmyeloablative dose (2 Gy) of TBI. The higher dosage of radiation used in the monkey study may have enhanced the engraftment of transduced stem cells by more-effective elimination of endogenous, untransduced stem cells. Differences in the packaging constructs and transduction con-

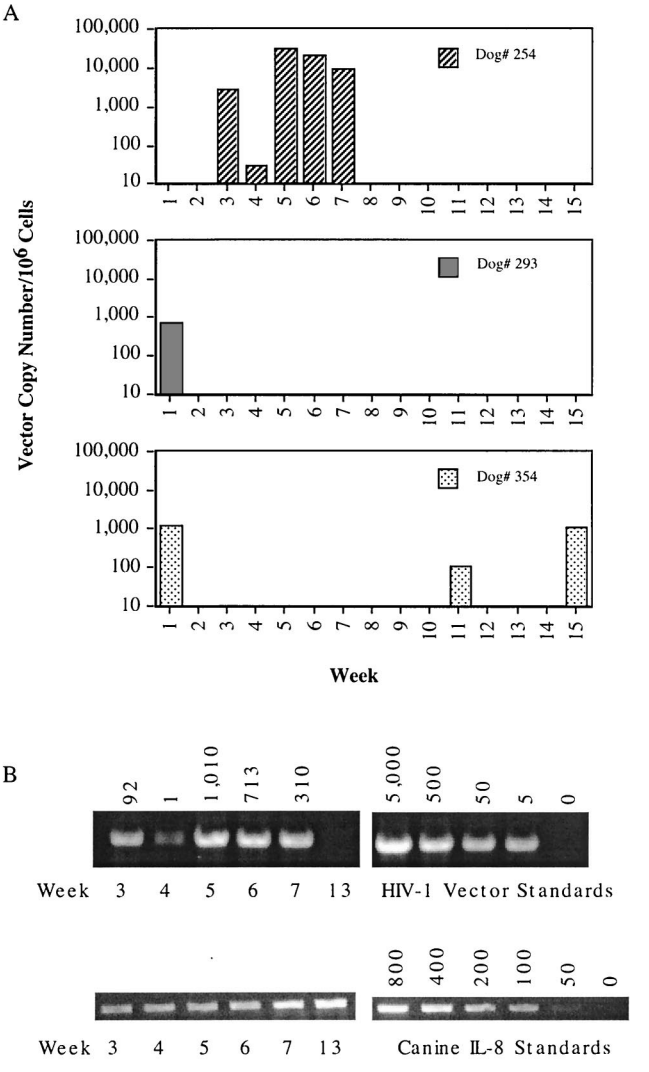

FIG. 4. Semiquantitative nested PCR analysis for vector sequences of peripheral blood MNC from dogs transplanted with autologous $CD34^+$ bone marrow cells transduced with HIV-1 and MoMLV vectors. (A) Results of PCR analyses of genomic DNA isolated from granulocytes in peripheral blood for HIV-1 vector sequences are shown for dogs 254, 293, and 354 as vector copy numbers per 10^6 cells. Samples were obtained weekly for 24 weeks, but only results for the first 15 weeks are shown, since no positive signals were obtained for later samples. (B) Ethidium bromide-stained gel from PCR of selected samples from dog 254. Numbers of weeks posttransplantation at which samples were obtained are shown at the bottom, and the calculated vector copy numbers per 200 ng of sample DNA are shown at the top. Known amounts of genomic DNA (0 to 5,000 proviral copies) from canine D17 cells transduced with an HIV-1 vector, along with 200 ng of carrier DNA derived from canine bone marrow MNC, were used in parallel (shown on the right) to derive the standard curve, with the "zero" sample containing 5,000 copies of HIV-1 vector plasmid. The primers were designed to preferentially amplify vector genomes following reverse transcription and not those from possible contaminating plasmid DNA. Note the absence of any PCR signal in the "zero" standard containing 5,000 copies of HIV-1 vector plasmid. The bottom panels show PCR amplification of a control canine IL-8 gene from 100 ng of each of the dog samples. Indicated amounts (0 to 800 ng) of genomic DNA from a canine thyroid adenocarcinoma cell line, CTAC, were used in the control PCR (bottom panel, right lanes).

ditions could also have contributed to the different outcomes of the two studies. The packaging construct used in the monkey study lacked Vpr, while the one used in the present study lacked Vpu. Vpr can produce apoptosis of target cells (27–29). Further studies are needed to determine if the accessory proteins produced by the packaging construct influence gene transfer into canine hematopoietic stem cells. The sudden disappearance of HIV-1 vector signals in dog 254 suggests the possibility of immune system-mediated elimination of transplanted cells. Cellular and humoral immune responses to EGFP after transplantation of transduced hematopoietic cells into rhesus macaques conditioned by nonmyeloablative TBI have been described previously (20). We are in the process of testing serum samples obtained from the animals for antibodies to EGFP by Western blot analysis. Further modifications to transduction conditions, use of alternative conditioning regimens, and immunosuppressive treatment after transplantation of transduced cells may lead to improved engraftment, persistence of transduced cells, and expression of transgene.

To summarize, we have shown that HIV vectors can transduce canine bone marrow MNC more efficiently than MoMLV vectors. Efficient transduction occurred with a single overnight exposure of freshly isolated bone marrow MNC to virus-containing supernatant in the absence of cytokine prestimulation. This is in contrast to our earlier experience with MoMLV vectors, which could transduce canine bone marrow MNC only after cocultivation of target cells with virus producer cells and/or after multiple infections in the presence of cytokines. We have also demonstrated that Tat72- and Tat86-encoding vectors are equally efficient for transduction of canine hematopoietic progenitor cells. Tat vectors may be useful not only for providing high levels of therapeutic gene expression in hematopoietic cells but also for study of the biological effects of Tat in those tissues in the canine model.

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